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Some relationships between membrane phospholipid domains, conformational order, and cell shape in intact human erythrocytes

David J. Moore [1,a], Scott Gioioso [a], Richard H. Sills [b], Richard Mendelsohn [a,*]

[a] *Department of Chemistry, Newark College of Arts and Sciences, Rutgers University, Newark, NJ 07102, USA*
[b] *Saint Barnabas Medical Center, Livingston, NJ 07039, USA*

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Abstract

A novel method developed in this laboratory [D.J. Moore et al., Biochemistry 35 (1996) 229–235; D.J. Moore et al., Biochemistry 36 (1997) 660–664] to study the conformational order and the propensity for domain formation of specific phospholipids in intact human erythrocytes is extended to two additional species. Acyl chain perdeuterated 1,2-dilauroylphosphatidylethanolamine ($diC_{12}PE\text{-}d_{46}$) was incorporated preferentially (in separate experiments) into the inner leaflet of stomatocytic erythrocytes and into the outer leaflet of echinocytic erythrocytes, while acyl chain perdeuterated 1,2-dipentadecanoylphosphatidylcholine ($diC_{15}PC\text{-}d_{58}$) was incorporated into the outer leaflet of echinocytic erythrocytes. The conformational order and phase behavior of the incorporated molecules were monitored through FT-IR studies of the temperature dependence of the CD_2 stretching vibrations. For both $diC_{12}PE\text{-}d_{46}$ and $diC_{15}PC\text{-}d_{58}$, the gel→liquid crystal phase transition persisted when these lipids were located in the outer leaflet of echinocytic cells, a result indicative of the persistence of phospholipid domains. In each case, the transition widths were broadened compared to the pure lipids, suggestive of either small domains or the presence of additional molecular components within the domains. The conformational order of $diC_{12}PE\text{-}d_{46}$ differed markedly depending on its location and the morphology of the cells. When located predominantly in the inner membrane of stomatocytes, the phase transition of this species was abolished and the conformational order compared with pure lipid vesicles at the same temperature was much lower. The current results along with our previous studies provide a sufficient experimental basis to deduce some general principles of phospholipid conformational order and organization in both normal and shape-altered erythrocytes.

Keywords: Membrane domain; Erythrocyte shape; FT-IR; Lipid conformational order; Deuterated phospholipid

1. Introduction

A variety of biophysical experiments, the majority utilizing fluorescence techniques, have demonstrated the existence of domains in biological membranes. While fluorescence techniques posses exquisite sensitivity for detection of domains, they do not provide molecular structure or conformational information about the domain constituents. In recent reports we have described a novel method which utilizes FT-IR spectroscopy and acyl-chain perdeuterated phospholipids to detect the conformationally sensitive CD_2 stretching vibrations of a specific phospholipid spe-

* Corresponding author. Fax: +1 (973) 353-1264; E-mail: mendelso@andromeda.rutgers.edu
[1] Present address: ISP Corporation, Wayne, NJ 07470, USA.

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cies within the lipid bilayer of an intact cell membrane [1,2]. In these studies perdeuterated phospholipids were incorporated into the plasma membrane of intact human erythrocytes and the conformational order of the incorporated lipid species was determined separately from the entire population of endogenous membrane phospholipids. Our first study described the method through a comparative study of acyl-chain perdeuterated dimyristoylphosphatidylcholine ($diC_{14}PC\text{-}d_{54}$) and acyl-chain perdeuterated dimyristoylphosphatidylserine ($diC_{14}PS\text{-}d_{54}$) [1]. The experiments suggested that $diC_{14}PC\text{-}d_{54}$ persisted in domains in the outer monolayer while $diC_{14}PS\text{-}d_{54}$ was dispersed in the inner monolayer. A subsequent study showed that $diC_{14}PS\text{-}d_{54}$ incorporated into the outer leaflet of echinocytic erythrocytes was conformationally ordered, whereas the same molecule translocated into the inner leaflet of stomatocytic erythrocytes was highly conformationally disordered [2].

The current report extends our previous investigations to phospatidylethanolamines (PE) and a longer chain phosphatidylcholine (PC). Neither PE nor PC are completely asymmetrically distributed in the erythrocyte membrane with PE being approximately 20% outer leaflet and 80% inner leaflet and PC being 80% outer leaflet and 20% inner leaflet. The current study examines 1,2-dilauroylphosphatidylethanolamine ($diC_{12}PE$) both in the inner leaflet of stomatocytic cells and trapped in the outer leaflet of echinocytic erythrocytes. In addition, the current report examines the conformational order of 1,2-dipentadecanoylphosphatidylcholine ($diC_{15}PC$) incorporated into the outer leaflet of echinocytic intact erythrocytes.

The current data, considered in combination with our earlier investigations, provide a sufficient experimental basis for some insights as to the general principles of phospholipid conformational order and organization in the erythrocyte membrane. Substantial differences are noted between the conformational properties and phase behavior of lipids located in the inner and outer monolayers.

2. Materials and methods

2.1. Materials

Acyl chain perdeuterated 1,2-dilauroylphosphatidylethanolamine ($diC_{12}PE\text{-}d_{46}$) and 1,2-dipentadecanoylphosphatidylcholine ($diC_{15}PC\text{-}d_{58}$) were custom synthesized by Avanti Polar Lipids (Alabaster, AL). The phase transition of these lipids were 4–5°C below that for pretreated species, as expected. All other chemicals, including glycerophosphoserine, were from Sigma (St. Louis, MO).

2.2. Erythrocytes

Human erythrocytes were collected from healthy adult volunteers by venipuncture into heparin. Cells were washed three times with phosphate-buffered saline (PBS: 138 mM NaCl, 5 mM KCl, 6.1 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgSO_4$, and 5 mM glucose, pH 7.4), and the buffy coat was removed.

2.3. Vesicle preparation and erythrocyte-vesicle incubations

These were carried out as described previously [1]. For the long incubation periods required with $diC_{12}PE$ and $diC_{15}PC$, the PBS was supplemented with additional glucose and penicillin. In all experiments a parallel control incubation (minus only the lipid vesicles) was included. Aminophospholipid translocase inhibition was performed as previously described [2].

2.4. Lipid incorporation

Incorporation of exogenous lipid varies in fairly unsystematic fashion from 1–3% of the total lipid. This is measured in two ways: (1) from the relative intensities of the CD_2 stretching modes to the CH_2 stretching modes in IR spectra of a total extract, and (2) by gas chromatography.

2.5. Erythrocyte morphology

Interference contrast microscopy (ICM) was used to examine erythrocyte morphology after incubation. The morphologic index (a qualitative marker of average cell shape) was determined as previously described [1,3]. Discocytes were given a score of 0, stomatocytes were scored from −1 to −4 and echinocytes from +1 to +5. The morpho-

logic index was determined from averaging 200 cells.

2.6. Sample preparation for FT-IR spectroscopy

After incubation, erythrocyte samples were packed by centrifugation and the supernatant removed. The erythrocytes were then washed with ten volumes of PBS and the cells separated from the vesicles by centrifugation for 3 min at $3000 \times g$. This washing process was then repeated several times if necessary, and, after removing the supernatant, the erythrocyte pellet was concentrated by spinning for 5 min at $10\,000 \times g$.

A major issue for the success of these experiments is the requirement that there be no vesicles remaining that 'stick' to the cells. Many control experiments has been undertaken to ensure that this is the case. For example, erythrocytes have been incubated for a relatively short time (so that no exogenous lipids have a chance to become incorporated into the cell membranes). These are then washed and the residual deuterium signal in the cells monitored. After an appropriate number of washes, no CD_2 stretching signal is observed in the IR. The same level of washing is used in the current experiments.

In addition, lipid species for which the phase transition is abolished show no signs of a residual melting process. Had there been significant populations of vesicles 'sticking' to the cells, we would have observed a normal phase transition for these. We did not. In addition, for DMPS confined to the outer monolayer (see [2]), the transition is also abolished. So it is not necessary for lipids to be targeted to the inner monolayer in order to abolish their phase transitions. These observations are consistent with no substantive 'sticking' of vesicles.

Vesicle-free washed erythrocyte samples are placed between two CaF_2 windows. Spectra were acquired on a Mattson RS-1 spectrometer by co-addition of 1024 interferograms collected from 4000 to 400 cm^{-1} at 4 cm^{-1} resolution. The interferograms were apodized with a triangular function and Fourier transformed with one level of zero filing. To minimize water vapor absorption, the spectrometer was continually purged with dry air. The CaF_2 windows enclosing all samples were held in a thermostatted transmission cell in which temperature was controlled with circulating water.

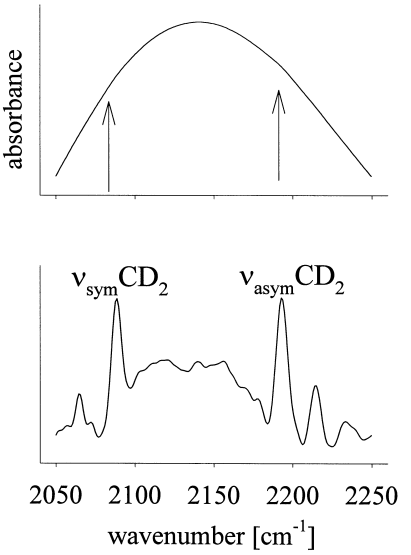

Fig. 1. Original (top) and inverted second derivative spectra (bottom) in the 2050–2250 cm^{-1} spectral region of intact erythrocytes into which $diC_{15}PC\text{-}d_{58}$ has been incorporated. The symmetric and asymmetric CD_2 stretching modes of the incorporated $diC_{15}PC\text{-}d_{58}$ are clearly visible in the second derivative spectrum.

3. Results

3.1. $DiC_{15}PC\text{-}d_{58}$

Erythrocytes incubated with $diC_{15}PC$ for 46 h became substantially echinocytic (stages 2,3, according to the semi-quantitative morphological scale of Daleke and Huestis [3,4]). The long incubation time required to induce this altered morphology is consistent with the low solubility of the monomer as the kinetic mechanism involves transfer of phospholipid monomers through the aqueous phase. As noted above, extra penicillin and glucose were added to the growth medium to ensure cell viability for this extended time period.

Fig. 1 displays representative FT-IR original and second derivative spectra for the CD_2 stretching region (2050–2250 cm^{-1}) of erythrocytes into which $diC_{15}PC\text{-}d_{58}$ has been incorporated. The original spectrum (top) displays extremely weak features at $\sim$2090 and 2195 cm^{-1}, arising from the symmetric (ν_{sym}) and asymmetric (ν_{asym}) CD_2 stretching vibrations of the incorporated species. As discussed in an earlier paper these peaks are clearly resolved using second derivative spectroscopy [1]. The thermotropic

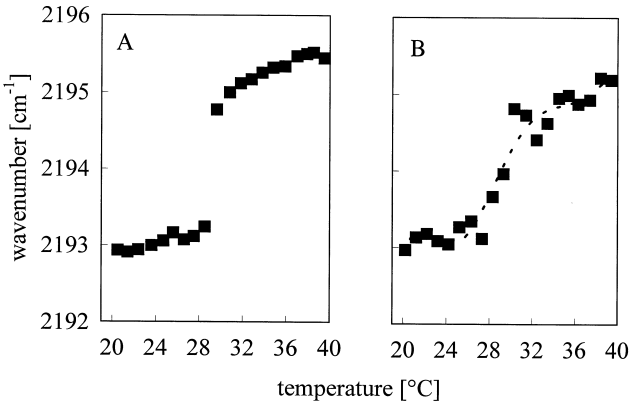

Fig. 2. (A) The thermotropic response of the asymmetric CD_2 stretching mode of $diC_{15}PC\text{-}d_{58}$ in pure vesicles. (B) The thermotropic response of the asymmetric CD_2 stretching mode of $diC_{15}PC\text{-}d_{58}$ incorporated into the outer membrane leaflet of intact erythrocytes.

behavior of $diC_{15}PC\text{-}d_{58}$ vesicles and $diC_{15}PC\text{-}d_{58}$ incorporated into erythrocytes are shown in Fig. 2A and B, respectively, as tracked by the temperature dependence of the asymmetric CD_2 stretching frequency ($\nu_{asym}CD_2$). The gel-liquid crystal phase transition for the pure lipid is evident at ~29°C. The transition temperature is lowered by about 4–5° from the pretreated species, an isotope effect consistent with observations for other phospholipids, and which probably reflects volume changes between CH_2 and CD_2 groups. The frequency increase of ~2 cm^{-1} at the transition reflects the introduction of conformational disorder (gauche rotations) into the acyl chains, although the quantitative relationship between either the absolute frequency or the change in frequency and the number of gauche rotamers introduced has remained elusive. It is evident from Fig. 2B, that a phase transition is still present in the echinocytic erythrocytes. However, the transition is broadened, occurring over a range of ~4°, compared with the pure vesicles, where it takes place over an interval of less than 1°C. Similar results were seen in each of five repeat experiments.

3.2. $DiC_{12}PE\text{-}d_{46}$

Erythrocytes incubated with $diC_{12}PE\text{-}d_{46}$ initially became echinocytic after a period of ~1 h. Incubations for up to 48 h revealed an increasing level of stomatocyctes (60–70% of the cells) formation ranging from stages 2–4, with a residual population of varying amounts of echinocytes and discocytes. Attempts to achieve a complete population of stomatocytes were unsuccessful. Cells incubated for substantially longer periods of time exhibited extensive lysis. These experiments were technically difficult, presumably because of the low solubility of the $diC_{12}PE\text{-}d_{46}$, the asynchronous behavior of the progressive shape changes within the cell population, and the inability to maintain cell viability for sufficiently long periods of time to achieve a homogeneous population of stomatocytes. In contrast, incubation of cells in such a

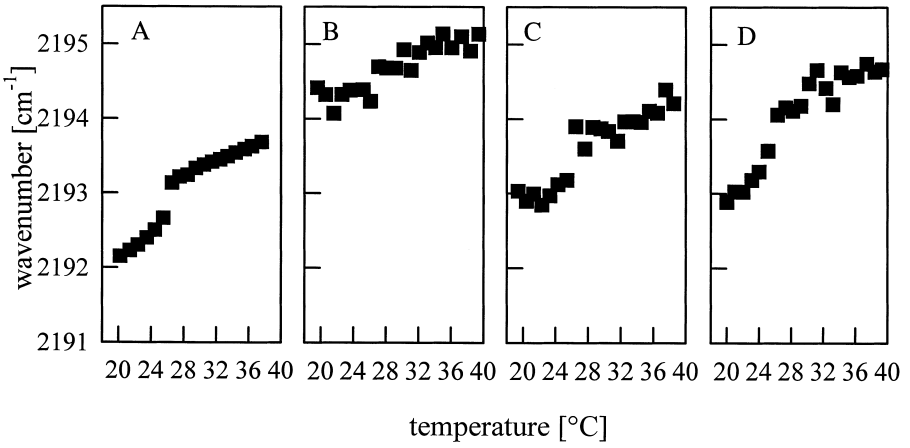

Fig. 3. (A) The thermotropic response of the asymmetric CD_2 stretching mode of $diC_{12}PE\text{-}d_{46}$ in pure vesicles. (B) The thermotropic response of the asymmetric CD_2 stretching mode of $diC_{12}PE\text{-}d_{46}$ in the inner membrane leaflet of intact erythrocytes (stomatocytes). The thermotropic response of the asymmetric CD_2 stretching mode of $diC_{12}PE\text{-}d_{46}$ trapped in the outer membrane leaflet of intact erythrocytes by inhibition of the aminophospholipid translocase with GPS (C) or by glucose deprivation (D).

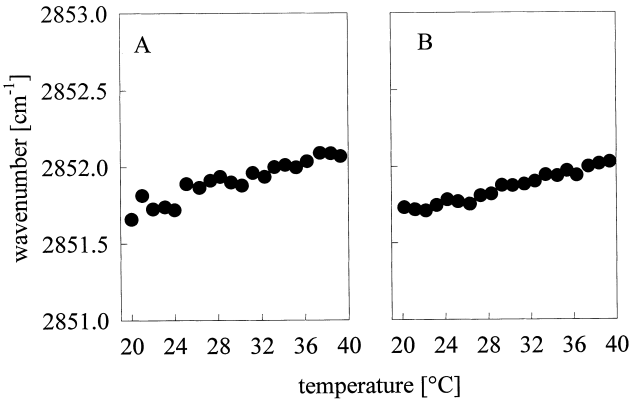

Fig. 4. The thermotropic response of the symmetric CH_2 stretching mode of all endogenous membrane phospholipids in echinocytic $diC_{15}PC\text{-}d_{58}$ incorporated erythrocytes (A) and stomatocytic $diC_{12}PE\text{-}d_{46}$ incorporated intact erythrocytes (B).

manner as to inhibit the aminophospholipid translocase produced a relatively homogeneous, stable population of echinocytes (stages 3,4) in a short time (1–4 h). This result was achieved independent of whether the translocation of PE was inhibited by omitting glucose from the incubation medium or by direct inhibition with glycerophosphoserine. No stomatocytes were formed in either circumstance for times up to 24 h.

The thermotropic behavior of the CD_2 asymmetric stretching frequency is shown for several preparations in Fig. 3A–D. The phase behavior of pure $diC_{12}PE\text{-}d_{46}$ vesicles is shown in Fig. 3A. The gel→liquid crystal phase transition is evident at ~26°C and is accompanied by a relatively small increase (0.4 cm^{-1}) in the frequency at the transition. The substantial slope of the frequency vs. temperature plot in both the gel and liquid crystalline phases suggest the significant non-cooperative formation of gauche rotations as the temperature is increased within each phase. The thermotropic behavior of $diC_{12}PE\text{-}d_{46}$ in stomatocytic red blood cells is shown in Fig. 3B. The phase transition evident in the pure vesicles has been completely abolished, and the frequency is increased at all temperatures compared the pure lipid. The variation with temperature is approximately linear. In contrast, the thermotropic behavior of $\nu_{asym}CD_2$ for $diC_{12}PE\text{-}d_{46}$ in echinocytic erythrocytes produced either by GPS inhibition of the aminophospholipid translocase (Fig. 3C) or by glucose deprivation of the cells (Fig. 3D), reveals a slight residual phase transition at 26°C. The frequency of the gel and liquid crystalline phases are increased from the respective phases in the pure vesicles in each case. Fig. 4 shows the thermotropic response of the symmetric CH_2 stretching frequency of the endogenous membrane phospholipids in $diC_{15}PC\text{-}d_{58}$ incorporated echinocytic cells and $diC_{12}PE\text{-}d_{46}$ incorporated stomatocytic erythrocytes. We were unable to monitor the asymmetric CH_2 stretching frequency (near 2920 cm^{-1}) in intact cells, since protein C-H modes interfere with the measurement. Thus membrane phospholipid conformational order can only be evaluated from the symmetric stretching mode. In pure lipids, the thermotropic responses of the symmetric and asymmetric modes invariably parallel each other. In Fig. 4, it is clear that the CH_2 stretching mode exhibits a monotonic increase as the temperature is raised without providing any evidence for a cooperative phase transition.

4. Discussion

In this study and in our previous investigations, we have developed a unique method for acquiring information about domain structure and conformational order of particular lipid species in the erythrocyte membrane. The method is based upon and extends the observations of Daleke and Huestis that characteristic shape changes are induced in the erythrocyte by the introduction of specific exogenous lipids into either or both of the erythrocyte monolayers [3]. The extent of the shape changes is readily followed with interference contrast microscopy and roughly correlates with the amount of exogenous lipid incorporated. Our extension of the method involves the incorporation of acyl chain perdeuterated phospholipids into the erythrocyte membrane followed by temperature-dependent FT-IR measurements of the CD_2 stretching frequencies from the deuterated chains to provide estimates of the conformational order and phase behavior of the incorporated species.

The current results for $diC_{12}PE$ and $diC_{15}PC$, taken in combination with our previous studies of $diC_{14}PC$ and $diC_{14}PS$, provide a reasonably complete picture of lipid conformational order and domain formation of the major species of the erythrocyte. The lipids were all selected on the basis of the avail-

ability of the perdeuterated analogue in order to provide a spectroscopic probe. In addition, they were required to have a phase transition at sufficiently low temperatures (but above 0°C) so that the proteins were not denatured at the high temperature end of the IR experiment. Although several experiments were attempted with diC_{10} lipids, these failed as the cells exhibited very rapid morphological alterations and formed spheres within a few minutes. We, therefore, had no control over the shape between a biconcave disk and a sphere.

There are several noteworthy aspects of the current experimental approach, the results of which are summarized in Table 1. The observation of a residual phase transition (which requires cooperative interactions between molecules for detection) in echinocytic erythrocytes containing $diC_{15}PC$ and $diC_{12}PE$ (current work) and $diC_{14}PC$ [1] provides direct evidence for the existence of domains of each of these species in the outer monolayer. Furthermore, it is noted that the cells are incubated at 37°C, well above T_m in each instance. The IR experiments are carried out by cooling pre-formed echinocytes to the desired starting temperature, then beginning the series of IR measurements. Thus, the domains of these species are either present over the entire range of temperatures studied, or form during the cooling following the incubation. The latter is considered less likely, since the unsaturated lipids of the outer monolayer are expected to have their phase transitions below 0°C, hence their miscibility properties with a small proportion of exogenous lipid should not be altered much between the incubation temperature (37°C) and the beginning of the IR measurement 15–20°C.

The observation of domains in echinocytes for two different lipid classes (PE, PC) and for two different chain lengths within a single lipid class ($diC_{14}PC$ and $diC_{15}PC$) suggests that the phenomenon may be quite general and not the result of our selection of a particular acyl chain length. The phase transitions in each instance are broadened from $< \sim 1°$ for the pure lipids to 3 or 4° for the incorporated PCs. This result is suggestive either of a relatively small domain size, or (more likely) a mixing of other membrane components into the domain.

The conformational order of lipids which normally reside, either preferentially (PE) or completely (PS), in the inner monolayer is altered when they are trapped in the outer leaflet by inhibition of the aminophospholipid translocator, either through ATP depletion or by direct GPS inhibition. The results of our current and prior studies are summarized in Table 1. The nature of the domains for $diC_{12}PE$ and $diC_{14}PS$ in the outer monolayer of echinocytes differ somewhat. As noted above, the former resembles a fairly pure lipid domain. In contrast, when $diC_{14}PS$ is localized in the outer monolayer of echinocytes, it exhibits no cooperative phase transition but manifests substantially higher conformational order ($\nu_{asym}CD_2$ ranging between 2193.0 and 2193.5 cm^{-1}) than when it is located in the inner monolayer of discocytic erythrocyte ($\nu_{asym}CD_2$ ranging between 2195.0 and 2195.9 cm^{-1}). The lack of a phase transition for this species when it is located in the outer

Table 1
Phase transition and conformational order data for various erythrocyte morphologies

Exogenous lipid	Cell morphology	Phase transition	Aminophospholipid translocase function	Conformational order relative to pure lipid vesicles	
				Below T_m	Above T_m
$DiC_{14}PC\text{-}d_{54}$	echinocytic	yes	normal	unchanged	unchanged
$DiC_{14}PC\text{-}d_{54}$/ $DiC_{14}PS$	discocytic	no	normal	reduced	increased
$DiC_{15}PC\text{-}d_{58}$	echinocytic	yes	normal	unchanged	unchanged
$DiC_{14}PS\text{-}d_{54}$	stomatocytic	no	normal	reduced	slightly increased
$DiC_{14}PS\text{-}d_{54}/DiC_{14}PC$	discocytic	no	normal	reduced	increased
$DiC_{14}PS\text{-}d_{54}$	echinocytic	no	GPS inhibited	unchanged	increased
$DiC_{14}PS\text{-}d_{54}$	echinocytic	no	inhibited by ATP deprivation	unchanged	increased
$DiC_{12}PE\text{-}d_{46}$	stomatocytic	no	normal	reduced	reduced
$DiC_{12}PE\text{-}d_{46}$	echinocytic	yes	inhibited by ATP deprivation	unchanged	unchanged
$DiC_{12}PE\text{-}d_{46}$	echinocytic	yes	GPS inhibited	unchanged	unchanged

monolayer was suggested to be the result of specific interactions between this lipid and other membrane components, such as the anion channel, which has been implicated in such interactions by the work of Glaser and his colleagues [5,6]. We suggest that it is more likely a lipid-protein rather than a lipid-lipid interaction that produces the observed thermotropic properties. If $diC_{14}PS$ were indeed miscible with other lipid constituents of the outer monolayer, we would anticipate more conformational disorder (rather than the ordering that was actually noted), since a high level of unsaturation is prevalent in the lipid population generally and in the outer monolayer in particular [7].

Analysis of the current IR results for $diC_{12}PE$ is complicated by the asymmetric distribution of PE across the erythrocyte membrane which is not absolute as it is for PS; 80% of the PE is estimated to reside in the inner monolayer in native discocytes. Whether this level of asymmetry is maintained in the stomatocytes is unknown but seems probable, especially in light of the work of De Jong and Ott [8], who showed that phospholipid asymmetry can be maintained in a system that does not contain an intact membrane skeleton or spectrin, i.e. conditions much more extreme than the current case. In addition, we were unable to prepare a population of cells which were completely stomatocytic.

The observed phase behavior of the incorporated species appears to be a strong function of cell morphology. We previously noted that incubation of erythrocytes with 1:1 mixtures of either $diC_{14}PS$-d_{54}/$diC_{14}PC$ or $diC_{14}PS$/$diC_{14}PC$-d_{54} permitted incorporation of the labeled lipid into the inner or outer monolayer respectively, while essentially preserving the initial biconcave disc shape of the erythrocyte [2]. Under these conditions, the deuterated lipid exhibits no phase transition, with $\nu_{asym}CD_2$ adopting values between those of the gel and liquid crystalline states. It is, therefore, tentatively suggested that the presence of domains in the exogenous deuterated component, as observed for $diC_{14}PC$-d_{54}, $diC_{15}PC$-d_{58} and $diC_{12}PE$-d_{46} is dependent on the echinocytic morphology. We further speculate that the exogenous lipids may be localized in the protrusions.

More generally, the availability of this novel IR method for monitoring the conformational order of particular lipid species offers several advantages. The conformational order of the particular species may show dramatic differences from the average order of the endogenous lipid population. For example, the conformational order of the entire lipid population of either stomatocytes or echinocytes, as sampled by the CH_2 stretching frequency, is presented in Fig. 4 and shows a relatively disordered membrane with no phase transitions between 15 and 45°C. A gradual increase in frequency occurs as the temperature is raised revealing a non-cooperative increase in the disorder. In contrast, our studies reveal that, when the individual species are sampled, large differences in order and phase transitions behavior may be observed.

Several extensions of the current approach are under investigation. These include investigating the lipid phase behavior and domain organization in normal membrane events as well as pathological samples such as sickle cell erythrocytes.

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